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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 9/98	A1	(11) International Publication Number: WO 91/09943 (43) International Publication Date: 11 July 1991 (11.07.91)

(21) International Application Number: PCT/DK90/00341

(22) International Filing Date: 21 December 1990 (21.12.90)

(30) Priority data:

6540/89	21 December 1989 (21.12.89)	DK
0847/90	5 April 1990 (05.04.90)	DK
0848/90	5 April 1990 (05.04.90)	DK

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(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: METHOD FOR CRYSTALLIZATION OF ENZYMES

(57) Abstract

The method for crystallization of enzymes is characterized by the fact that an aqueous enzyme containing liquid with a relatively high enzyme purity and with a concentration of pure enzyme protein of at least 5 g/l of enzyme containing liquid is used as a starting material, and that a crystallization agent, which is an easily soluble salt of the non-halide type is added to the starting material to a final concentration which is considerably smaller than the concentration needed to precipitate the enzymes in an amorphous form. The method is simple and cheap, and it is compatible to industrial requirements.

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METHOD FOR CRYSTALLIZATION OF ENZYMES

The invention encompasses a method for crystallization of enzymes.

Enzymes are usually provided as liquids or solid materials for industrial purposes. When not provided as liquids, they are usually provided as amorphous materials, because the known methods for crystallization of enzymes usually are regarded as too expensive to be used in an industrial scale.

Thus, the purpose of the invention is the provision of a method for crystallization of enzymes, which is simple and cheap, and which is compatible to industrial requirements.

The method according to the invention is characterized by the fact that an aqueous enzyme containing liquid with a relatively high enzyme purity and with a concentration of pure enzyme protein of at least 5 g/l of enzyme containing liquid is used as a starting material, and that a crystallization agent, which is an easily soluble salt of the non-halide type is added to the starting material to a final concentration which is smaller than the concentration needed to precipitate the enzymes in an amorphous form.

When carried out on an industrial scale, the crystals are separated in a filter, and the crystals are subsequently flushed for purification purposes. Reference is made to Fig. 1.

In this specification with claims it is to be understood that an easily soluble salt is a salt, which in pure water at 25°C exhibits a solubility above 5 g/l.

The above indicated starting materials are known in the art, and they can be provided for instance as described in chapter 9 ("Production of Microbial Enzymes") in Microbial Technology, 2nd edition, Vol. 1, Academic Press, Inc., 1979; and thus, an outline of recovery and purification methods for technical grade enzymes can be found in this chapter. Furthermore US patent No. 3,795,583 describes purification methods for enzyme containing culture broths.

Surprisingly it has been found that the method according to the invention which is simple and cheap, can be carried out with a yield of up to 95%, and that it can easily be adopted to industrial practice.

In a preferred embodiment of the method according to the invention the enzyme purity is above 20% (i.e. the pure enzyme amounts to more than 20% of the total dry matter in the enzyme containing liquid), and the crystallization agent is added to the starting material to a final concentration corresponding to an added amount of crystallization agent of from 0.02 to 1.7M of crystallization agent, preferably from 0.05 to 1.6M, more preferably from 0.10 to 1.5M.

Thus, the preferred starting material for the method according to the invention for crystallization of enzymes is an aqueous enzyme containing liquid with an enzyme purity of above 20% (i.e. the pure enzyme amounts to more than 20% of the total dry matter in the enzyme containing liquid), and with a concentration of enzyme protein of at least 5 g/l of enzyme containing liquid. The time necessary for crystallization is usually between 5 and 12 hours. By addition of crystal seeds in an amount of e.g. 1% the crystallization velocity can be accelerated. The crystals are preferably separated from the supernatant by filtration.

In a preferred embodiment of the method according to the invention an aqueous enzyme containing liquid with a relatively high enzyme purity and with a concentration of pure enzyme protein of at least 5 g/l of enzyme containing liquid and with a temperature between 15-45°C, preferably 20-45°C is used as a starting material, and a crystallization agent, which is an easily soluble salt is added to the starting material to a final concentration which is smaller than the concentration needed to precipitate the enzymes in an amorphous form.

In a preferred embodiment of the method according to the invention the enzyme purity is above 20% (i.e. the pure enzyme amounts to more than 20% of the total dry matter in the enzyme containing liquid), and the crystallization agent is added to the starting material to a final concentration corresponding to an added amount of crystallization agent of from 0.02 to 1.7M of crystallization agent, preferably from 0.05 to 1.6M, more preferably from 0.10 to 1.5M.

In a preferred embodiment of the method according to the invention the enzyme is a protease, lipase, amylase, cellulase, hemicellulase, pectinase, amidase or oxidase. These enzymes are commonly used as additives in detergents, and thus, when crystalline enzymes are desirable in the detergent

industry, this embodiment is preferred. Also protein engineered variants of these enzymes are within the scope of the invention.

In a preferred embodiment of the method according to the invention the enzyme is a protease and the protease is a Subtilisin type protease, preferably Savinase® or a protein engineered variant of Savinase® or Alcalase®. Other examples of such proteases are Alcalase®, Subtilisin NOVO or protein engineered variants of these, which are very commonly used as additives in detergents, and thus, when crystalline Savinase®, Esperase®, Alcalase®, Subtilisin NOVO or protein engineered variants of these is desirable in the detergent industry, this embodiment is preferred.

In a preferred embodiment of the method according to the invention the crystallization agent is Na, K, Ca, or Mg formate, acetate or nitrate. In this manner a rapid and efficient crystallization is performed.

DK patent application No. 872/86 describes a method for crystallization of an enzyme containing solution, wherein a supersaturated solution is crystallized at a pH close to the isoelectric point of the enzyme.

US 4,699,882 describes a crystallization method specifically directed to glucose isomerase. One of the imperative steps is cooling to about 16°C or below. Another is addition of ammonium and/or magnesium sulphate.

WO 89/08703 describes a Subtilisin crystallization process, which as an imperative step uses a crystallization agent, which is a halide salt, and which can only be performed satisfactorily below around 10°C.

Thus, all these prior art methods differ substantially from the method according to the invention.

EXAMPLES 1 - 4

The starting material for the crystallization of Savinase® crystals is prepared in the following manner.

To 1 kg of Savinase® fermentation broth (US patent No. 3,723,250) are added 7.5 g of $CaCl_2 \cdot 2H_2O$ and 1 litre of water. The pH is adjusted to 8.0 with an aqueous sodium hydroxide solution. The suspension is then flocculated by addition of Superfloc C 521 flocculation agent (approx. 15 g/litre of broth) and Superfloc A 130 flocculation agent (approx. 0.2 g/litre of broth). The flocculated suspension is centrifuged and the supernatant is filtered on an appropriate filter sheet in order to obtain a clear liquid. The filtrate is concentrated by evaporation to a value of RI (refractive index) of 12% and then heated to approx. 38°C. At this temperature sodium sulphate (250 g/kg enzyme solution) is added stepwise to the solution with rapid mixing.

The precipitate is filtered off and is reslurried in cold (< 5°C) water (3-5 litres of water/kg of filter cake). The undissolved material is removed by filtration and the clear filtrate is ultra- and diafiltered to a point where the value of the RI (Refractometer Index) dry matter in the permeate is less than 0.5% and the value of the RI dry matter in the concentrate is between 10-16%. The proteolytic activity is 18-22 KNPU/g.

The resulting concentrate exhibits a content of pure Savinase® protease on a dry matter basis above 25% w/w.

To the starting material, i.e. the above indicated concentrate, is added 7% of calcium formate (0.54 M added) serving as a precipitation agent, at 20-25°C and a pH value of approximately 5.0. The mixture is seeded with Savinase® crystals (approx. 1% w/w) and then gently agitated, and after 3-6 hours a powerful precipitate of crystalline Savinase® can be observed. The amount of the crystal sludge is approx. 20% of the total volume and exhibits an enzyme activity of approx. 86 KNPU/g. The precipitation yield on the basis of KNPU is around 81%.

The following Examples 2 - 4 were carried out with the same Savinase® concentrate, seeding procedure, temperature (20-24°C) and pH (5.0). All the generated crystal sludges had an activity of about 80 KNPU/g.

The variable parameters in Examples 2 - 4 appear from the following table.

Table 1

Example no.	Precipitation agent	Concentration of added precipitation agent	% sludge	% Yield
2	$Ca(CHOO)_2$	0.38 M	17.5	73
3	$K(CH_3COO)$	0.82 M	15.0	74
4	$K(CH_3COO)$	1.00 M	16.0	78

EXAMPLES 5 - 69

It has been found that the crystallization process is dependent on the following parameters:

pH: with increasing pH the crystal size decreases, and the crystallization velocity increases.

Salt concentration: with increasing salt concentration the crystallization yield increases, the crystallization velocity increases, and the crystal size decreases.

Salt type: the crystallization performance varies from salt to salt.

Temperature: with increasing temperature the crystallization velocity increases, and the crystal size decreases.

Enzyme concentration: with increasing enzyme concentration the crystallization velocity increases, the crystallization yield increases, and the crystal size decreases.

Enzyme purity: with increasing enzyme purity the crystallization velocity increases, and the crystallization yield increases.

The following examples 5 - 18, which illustrate the above indicated pH dependency and the salt concentration dependency, were carried out with a Savinase® concentrate, made by the method mentioned in Examples 1 - 4. The temperature was kept at 20-25°C and the concentration of enzyme was approx. 55 g/l. The crystallization agent was potassium acetate. The enzyme purity was approximately 35%.

Example no.	pH	Mol potassium acetate added/l	crystallization start, comment
5	4.0	0.8	no crystallization
6	4.5	0.8	1-2 h, 20-50 μm crystals
7	5.5	0.8	½-1 h, 10-50 μm crystals
8	6.5	0.8	≈ ½ h, 5-50 μm crystals
9	7.5	0.8	≈ ½ h, 5-10 μm crystals

Example no.	pH	Mol potassium acetate added/l	crystallization yield, comment
10	5.5	0.05	no crystallization
11	5.5	0.10	no crystallization
12	5.5	0.20	no crystallization
13	5.5	0.40	≈ 75%, 10-30 μm crystals
14	5.5	0.60	≈ 84%, 5-20 μm crystals
15	5.5	0.80	≈ 84%, 1-20 μm crystals
16	5.5	1.20	≈ 84%, 1-20 μm crystals
17	5.5	1.40	≈ 87%, 1-10 μm crystals
18	5.5	>1.60	amorphous fragments

Reference is made to Fig. 2, which shows crystallization performance (Savinase®) as a function of enzyme purity. Fig. 2 shows that the crystallization velocity and the crystallization yield increases with increasing enzyme purity.

The following examples 19-25 which show the crystallization performance as a function of enzyme concentration, were carried out at 20-25°C, with 0.8 mol added potassium acetate/l and at pH 4.9. The enzyme purity is about 38% (DS basis). The Savinase® samples are taken from different stages in the final ultrafiltration in the recovery process mentioned in Examples 1 - 4, corresponding to the values in the column "g enzyme/l".

Example no.	g enzyme/l	Start of crystallization	Yield
19	8.6	÷	0%
20	17.0	2-3 h	70%
21	25.0	1½-2 h	85%
22	33.0	1½ h	88%
23	41.0	½-1 h	90%
24	50.0	½-1 h	92%
25	58.0	½-1 h	94%

The following examples 26-29 which show the crystallization performance as a function of enzyme temperature, are carried out with 0.51 mol added potassium acetate/l, pH 5.5 and an enzyme concentration at about 55 g/l. The enzyme purity is about 36% (DS basis). Maximum crystallization time: 12 hours.

Example no.	Temperature	Start of crystallization	Yield
26	10°C	> 10 hours	< 1%
27	15°C	3 hours	78%
28	20°C	2 hours	85%
29	30°C	¾ hours	93%

The following examples 30 - 69 show that the crystallization process can easily be used with other subtilisins such as Alcalase® and Durazym®.

The starting material for the crystallization is prepared by a recovery process which is very similar to that indicated in Examples 1 - 4.

In the case of Alcalase®, during the flocculation step besides $CaCl_2$ about 4 g of $NaAlO_2$ per l of fermentation broth is added. The product will end up with a proteolytic activity at about 3.5 AU/g.

Durazym®, which is a protein engineered version of Savinase®, is prepared analogously to the crystallization of Savinase® in Examples 1 - 4. The product will end up with a proteolytic activity at about 19 DPU/g.

Before crystallization both enzymes have an enzyme purity above 25% measured on dry matter basis.

In the following examples 30-34 the enzyme is Alcalase®, and the pH during the crystallization is 4.5-8.5, the temperature is between 20 and 25°C, the crystallization agent is sodium formate, which is added in a concentration of 1.5 M/l, the enzyme concentration is approximately 66 g/l, and the enzyme purity is 50%.

Example no.	pH	Start of crystallization	Yield
30	4.5	1 hour	92%
31	5.5	1 hour	93%
32	6.5	½ hour	95%
33	7.5	½ hour	≈ 93%
34	8.5	« ½ hour	≈ 93%

In the following examples in which Alcalase® and different crystallization agents are used the pH during the crystallization is between 4.5 and 5.0, the temperature is between 20 and 25°C, the crystallization time is between 6 and 24 hours, the enzyme concentration is approx. 66 g/l and the enzyme purity is 50%.

Enzyme: Alcalase®

Example no.	Salt	M salt added/l	Yield
35	$Na(CH_3COO)$	0.7	35%
36	$Na(CH_3COO)$	1.2	70%
37	$Na(CH_3COO)$	1.7	78%
38	$Na(CHOO)$	0.9	80%
39	$Na(CHOO)$	1.1	90%
40	$Na(CHOO)$	1.4	96%
41	$Ca(CHOO)_2$	0.4	45%
42	$Ca(CHOO)_2$	0.8	60%
43	$Ca(CHOO)_2$	0.9	78%

In the following examples which are performed with Alcalase® and with crystallization with sodium formate at different enzyme concentrations the pH during the crystallization is between 4.5 and 5.0, the temperature is between 20 and 25°C, the crystallization time is 12 hours (at 20 to 25°C) plus 12 hours at 10°C. The enzyme concentration is approx. 66 g/l, the enzyme purity is 50%, and the salt is added in a dosage of 1.5 Mol/l.

Example no.	g enzyme/l	Start of crystallization	Yield
44	19	2 hours	60%
45	29	1 hour	81%
46	40	1 hour	86%
47	50	¾ hour	90%
48	58	< ¾ hour	93%

The following examples with Alcalase® which illustrate crystallization performance as a function of temperatures are carried out at pH 4.5 and a temperature between 5 and 30°C. The sodium formate is added in an amount of 1.5 Mol/l. The enzyme concentration is approx. 66 g/l and the enzyme purity is 50%. The crystallization time is 12 hours at the temperature specified plus 12 hours at 10°C.

Example no.	Temperature	Start of crystallization	Yield
49	5°C	> 12 hours	-
50	15°C	> 12 hours	-
51	20°C	1 hour	90%
52	25°C	< ½ hour	≈ 88%
53	30°C	< ½ hour	≈ 86%

The following examples which illustrate crystallization performance of Alcalase® as a function of enzyme temperature are carried out at pH 5.0 and at a temeprature between 15 and 30°C. The dosage of calcium formate is 0.8 Mol added /l. The enzyme concentration is approx. 63 g/l and the purity is 50%.

The crystallization time is 12 hours (at the specified temperature) plus 12 hours at 10°C.

Example no.	Temperature	Start of crystallization	Yield/crystal size
54	15°C	10 hours	4% / 50-80 μm
55	20°C	4 hours	16% / 40-80 μm
56	25°C	2¼ hour	46% / 10-60 μm
57	30°C	< ½ hour	49% / 15-40 μm

The following examples which illustrate crystallization of Alcalase® at different pH values the pH is varied from 4.5 to 8.5, the temperature is between 20 and 25°C, the salt dosage is 0.8 Mol added/l the enzyme concentration is approx. 66 g/l and the enzyme purity is 50%. Salt: pottassium acetate.

Example no.	pH	Start of crystallization	Yield/crystal size
58	4.5	3½ hour	55% / 30-70 μm
59	5.5	1½ hour	≈ 60%*) / 1-10 μm
60	6.5	1½ hour	≈ 60%*) / 1-10 μm
61	7.5	1 hour	≈ 60%*) / 1-10 μm
62	8.5	¼ hour	≈ 60%*) / 1-10 μm

*) The crystals are very small and therefore hard to separate from the mother liquor

The following examples with Durazym®, which show crystallization with different crystallization agents are carried out at pH 4.9 and a temperature between 20 and 25°C. The enzyme concentration is approx. 48 g/l and the enzyme purity is approx. 30%.

Enzyme: Durazym®

Example no.	Salt	M salt added/l	Yield
63	Na(CHOO)	0.6	42%
64	Na(CHOO)	1.1	90%
65	Na(CHOO)	1.5	96%
66	$K(CH_3COO)$	0.6	62%
67	$K(CH_3COO)$	0.8	83%
68	$K(CH_3COO)$	1.2	86%

EXAMPLE 69

Crystallization of *Candida* lipase B

Candida lipase B is described in WO 88/02775, from which is appears that this lipase can be produced by means of *Candida antarctica* DMS 3855.

After fermentation the culture broth is pretreated by addition of 0.25 l of water per liter of culture broth. pH is adjusted to 8.0 by means of an aqueous solution of NaOH. The suspension is filtered on a drum filter, which is precoated with Dicalite 4208 kiselguhr. The filtrate is subsequently filtered on appropriate filter plates in order to obtain a completely clear liquid. Finally an ultrafiltration and a diafiltration is carried out until a value of RI (Refrectometer Index) dry substance in the concentrate of around 12% and until the value of RI dry substance in the permeate is less than 2%. Finally a sterile filtration is carried out. This filtrate which

exhibits a lipase activity of approx. 25 KLU/g and a pH of 7.0 is the starting material for the crystallization. To the starting material is added 4-6% salt, which is CH_3COOK(KAc) or HCOONa. After a few minutes a powerful precipitate of crystalline *Candida* lipase B is formed, and 2-5 hours later the crystalline precipitate amounts to around 20% of the total volume. The crystals exhibit an activity of 65-70 KLU/g, and the yield exceeds 80%. The crystals are small and needle shaped.

CLAIMS

1. Method for crystallization of enzymes, characterized by the fact that an aqueous enzyme containing liquid with a relatively high enzyme purity and with a concentration of pure enzyme protein of at least 5 g/l of enzyme containing liquid is used as a starting material, and that a crystallization agent, which is an easily soluble salt of the non-halide type is added to the starting material to a final concentration which is smaller than the concentration needed to precipitate the enzymes in an amorphous form.

2. Method according to Claim 1, wherein the enzyme purity is above 20% (i.e. the pure enzyme amounts to more than 20% of the total dry matter in the enzyme containing liquid), and the crystallization agent is added to the starting material to a final concentration corresponding to an added amount of crystallization agent of from 0.02 to 1.7M of crystallization agent, preferably from 0.05 to 1.6M, more preferably from 0.10 to 1.5M.

3. Method for crystallization of enzymes, characterized by the fact that an aqueous enzyme containing liquid with a relatively high enzyme purity and with a concentration of pure enzyme protein of at least 5 g/l of enzyme containing liquid and with a temperature between 15-45°C, preferably 20-45°C is used as a starting material, and that a crystallization agent, which is an easily soluble salt is added to the starting material to a final concentration which is smaller than the concentration needed to precipitate the enzymes in an amorphous form.

4. Method according to Claim 3, wherein the enzyme purity is above 20% (i.e. the pure enzyme amounts to more than 20% of the total dry matter in the enzyme containing liquid), and the crystallization agent is added to the starting material to a final concentration corresponding to an added amount of crystallization agent of from 0.02 to 1.7M of crystallization agent, preferably from 0.05 to 1.6M, more preferably from 0.10 to 1.5M.

5. Method according to Claims 1 to 4, characterized by the fact, that the enzyme is a protease, lipase, amylase, cellulase, hemicellulase, pectinase, amidase or oxidase.

6. Method according to Claim 5, characterized by the fact that the enzyme is a protease and that the protease is a Subtilisin type protease, preferably Savinase® or a protein engineered variant of Savinase®, or Alcalase®.

7. Method according to Claims 1 to 6, characterized by the fact that the crystallization agent is Na, K, Ca, or Mg formate, acetate or nitrate.

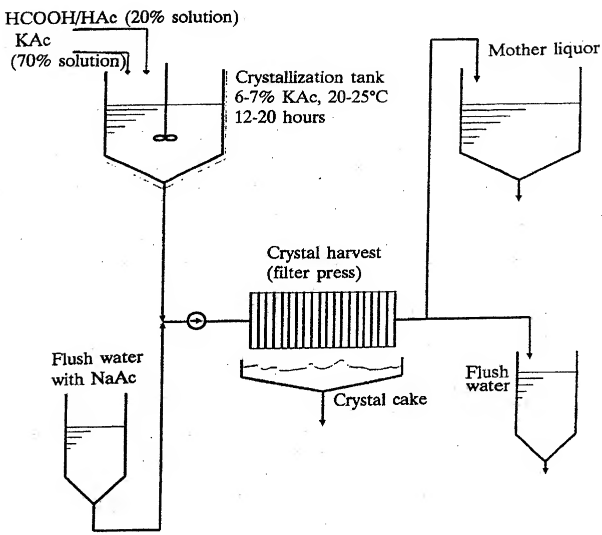
HCOOH/HAc (20% solution)
KAc
(70% solution)
Crystallization tank
6-7% KAc, 20-25°C
12-20 hours
Mother liquor
Crystal harvest
(filter press)
Flush water
with NaAc
Flush
water
Crystal cake

Fig. 1

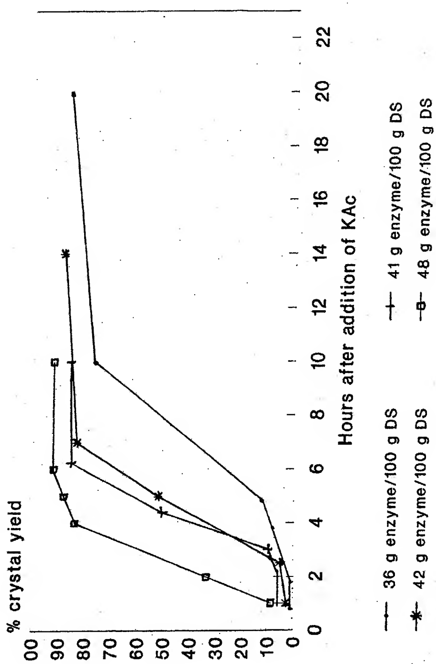
% crystal yield
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
Hours after addition of KAc
36 g enzyme/100 g DS
41 g enzyme/100 g DS
42 g enzyme/100 g DS
48 g enzyme/100 g DS

Fig. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 90/00341

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 9/98

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	WO, A1, 8908703 (GENENCOR, INC.) 21 September 1989, see the whole document --	1-6
X	DE, A, 1959603 (ELI LILLY AND COMPANY) 19 November 1970, see the whole document --	1-6
X	US, A, 4699882 (KALEVI J. VISURI) 13 October 1987, see the whole document --	1-6

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* Special categories of cited documents: [10]

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
3rd April 1991	1991 -04- 05
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Jack Hedlund Jack Hedlund

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 90/00341

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 91-02-28
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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